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ORIGINAL ARTICLE

Anti-hyperalgesic effect of CaMKII inhibitor is associated with downregulation of phosphorylated CREB in rat spinal cord

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Abstract

Purpose Calcium/calmodulin-dependent protein kinase (CaMK) II and its downstream effector cyclic adenosine monophosphate (cAMP) responsive element binding protein (CREB) may be involved in the development of neuropathic pain. The aim of this study was to examine the effect of the CaMKII inhibitor AIP on the association of CaMKII and CREB in a partial sciatic nerve ligation neuropathic pain model in rats.

Methods Male Sprague-Dawley rats were randomly divided into six groups: Sham operation; partial sciatic nerve ligation (SNL); AIP administration prior to or after SNL; and normal saline (NS) administration prior to or after SNL. NS or 10 μmol/L AIP (10 μl) were injected intrathecally in the NS group and AIP group, respectively, 20 min before or 4 days after nerve injury. The mechanical withdrawal threshold of the hindpaw was measured before and after SNL. In another cohort treated as above, the lumbar spinal cord was removed on post-surgery days 1, 3, and 7 to detect the expression of phosphorylated CaMKII (pCaMKII) and phosphorylated CREB (pCREB), by in situ immunostaining and western blot, respectively.

Results AIP significantly suppressed tactile allodynia in the SNL rats, and its effects lasted for 3 days when given prior to nerve injury. In contrast, it had a transitory effect when given after nerve injury. AIP decreased the expression of pCaMKII and pCREB and its effect was sustained for up to 3 days after the experiments.

Conclusion Intrathecal injection of a CaMKII inhibitor attenuated neuropathic pain. This benefit may have been due to the downmodulation of its downstream effector pCREB.

Keywords Neuropathic pain · Calcium/calmodulin-dependent protein kinase (CaMK) II · cAMP responsive element binding protein (CREB) · CaMKII inhibitor · Rat

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Introduction

Central sensitization is one of the mechanisms responsible for long-standing neuropathic pain, a term which refers to the increased activity resulting from synaptic plasticity established in somatosensory neurons in the dorsal horn of the spinal cord following peripheral noxious stimuli [1]. This heightened synaptic plasticity leads to a reduction in the pain threshold, an amplification of pain synaptic transmission, and a spread of pain sensitivity to non-injured areas. Calcium/calmodulin-dependent kinase II (CaMKII) has been widely implicated in synaptic plasticity, and it contributes to the long-term potentiation of C-fibers [2, 3] and the form of central sensitization in a rat model of neuropathic pain [4–6]. Intracellular protein kinases including CaMKII are capable of activating phosphorylated cyclic adenosine monophosphate (cAMP) responsive element binding protein (pCREB), in the spinal dorsal horn [7, 8]. Subsequently, pCREB can further initiate the

transcription of genes and their translation that may ultimately enhance synaptic plasticity, which is involved in the process of persistent pain development.

Critical amino acid residues of AIP (KKALRRQEAVDAL) is a highly specific inhibitory peptide of CaMKII. It has been shown that the long-term potentiation induced by sciatic nerve high-frequency electric stimulation in the spinal cord can be inhibited by the administration of AIP in rats [9] and hence it may have anti-nociceptive properties. The aim of the present study was to investigate the role of CaMKII/pCREB in the anti-hyperalgesic effect of AIP in a neuropathic pain model in rats.

Materials and methods

Animals

Male Sprague-Dawley rats (weight 250–280 g) were obtained from the Experimental Animal Center, Xuzhou Medical College, Jiangsu, China. All rats were housed in a room with lights on from 8:00 to 20:00 at 25 ± 2°C and a relative humidity of 55 ± 5%. A standard pellet diet was available from 14:00 to 23:00 with drinking water available ad lib. The rats fasted for 12 h before experiments. The study protocol was approved by the Animal Care and Use Committee of Gongli Hospital and followed the Guidelines for the Care and Use of Laboratory Animals.

Surgical procedures

After the baseline pain threshold was assessed with von Frey filaments (North Coast Medical, Gilroy, CA, USA) (see below), rats were randomly divided into four groups ($n = 8$/each): Sham surgery group, partial sciatic nerve ligation (SNL) group, SNL + AIP administered group and SNL + saline administered group. Surgery was performed under general anesthesia with 1.5% isoflurane in oxygen-rich air. Briefly, the left (ipsilateral) sciatic nerve was exposed just above its trifurcation and 1/3–1/2 of the nerve was ligated with a 6–0 non-absorbable silk suture [10]. In sham animals, the sciatic nerve was exposed in the same way but without ligation. For intrathecal administration of 10 μl AIP (10 μmol/L) or 10 μl saline, rats were immobilized with 1.6% isoflurane in oxygen and correct placement of the needle puncturing into the subarachnoid space through lumbar 5–6 interspace was established by reflex tail movement 30 min before surgery or 4 days after SNL surgery [11]. All animals ($n = 8$) were allowed to recover for 24 h and then their pain thresholds were assessed for a further 14 days.

Pain threshold assessment

The pain thresholds indicated by the mechanical withdrawal threshold (MWT) were assessed with a series of calibrated von Frey filaments (range from 2 to 15 g) by an investigator who was blinded to the experimental protocol. Briefly, animals were placed on a mesh floor and covered with an individual Plexiglass testing chamber (23 × 18 × 14 cm) for 15 min habituation prior to testing. A filament was applied when the rat was stationary and standing on all four paws and the application was maintained for approximately for 2 s. The filaments were applied perpendicularly to the plantar surface of the left hind paw with sufficient force to bend the filaments into an “S” shape for 6–8 s, and quick withdrawal or licking of the paw were regarded as positive reactions. The filaments were applied in increasing forces from 2 to 15 g until a response was finally detected from the animal in a series of four applications at 30 s intervals, then the 50% detection threshold was measured. The MTV that was consistent in three consecutive trials with each scale of filament was considered reliable and was used for statistical analysis as described previously [12].

Tissue harvesting

The 1st cohorts ($n = 4$/group/time point) described as above were deeply anesthetized with pentobarbital and perfused transcardically with 0.9% heparinized saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer on post-surgery days 1, 3, and 7. The whole spinal cord was removed by high-pressure injection via the vertebral canal, and the segment of lumbar enlargement (L4–L6) of the spinal cord was harvested for in-situ immunostaining for pCaMKII. The 2nd cohorts ($n = 3$/group/time point) described as above were deeply anesthetized with pentobarbital and then the segment of lumbar enlargement (L4–L6) of the spinal cord was harvested immediately by a surgical approach and kept at −80°C for western blot of pCREB.

Immunohistochemistry

Cryostat sections (30 μm) of the lumbar enlargement of the spinal cord were immunostained by a floating method. Briefly, after preincubation with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase, the sections were rinsed in phosphate-buffered saline (PBS) and then incubated with Anti-pCaMKII (1:100; Cell Signaling, Danvers, MA, USA) for 24 h at 4°C. Following incubation, tissue sections were washed three times with PBS and incubated with the biotinylated secondary antibodies for 1 h and then incubated with the avidin–biotin–peroxidase complex (Vectastain Elite ABC-Kit; Vector

Laboratories, Peterborough, UK) for 30 min at room temperature. After the sections were washed with PBS, the peroxidase reaction was developed by incubating the section in 0.02% 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) solution containing 0.003% hydrogen peroxide. Finally, the sections were then washed in PBS, mounted on slides, air-dried, dehydrated through a gradient of ethanol solutions (70–100%), cleared in xylene, and coverslipped. Quantitative image analysis for relative optical density of phospho-CaMKII in the superficial laminas of the dorsal horn was performed using imagepro-plus 6.0 software (Media Cybernetics, Bethesda, MD, USA). Six sections were randomly collected from each animal for data analysis.

Western-blot

Samples were homogenized in lysis buffer (5 mM Tris–HCl, 1 mM phenylmethylsulfonyl fluoride, 150 mM NaCl, 1% NP-40, 0.5% Na deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and protease and phosphatase inhibitors; pH 7.5). The homogenates were centrifuged for 15 min at 12,000*g* at 4°C. The quantity of protein in the supernatants was determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Protein extracts (30 µg per sample) were denatured in Laemmli sample loading buffer (10% SDS, 0.1 M TRIS pH 8.0, 50 mM dithiothreitol [DTT], 3 mM ethylenediaminetetraacetate [EDTA], 0.001% bromphenol blue) at 100°C for 5 min, separated by 15% SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). After blocking with 5% skim milk in TBS-T (0.1% Tween 20, 20 mM Tris–HCl, 137 mM NaCl, pH 7.6) for 2 h, the membrane was incubated with anti-pCREB-Ser^{133} antibody (1:1,000; Cell Signaling) or anti-actin antibody (1:50,000; Cell Signaling) in TBS-T overnight at 4°C. Subsequently, the membranes were incubated for 2 h with goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham Biosciences, Buckinghamshire, UK) at room temperature. The membranes were treated with an enhanced chemiluminescence (ECL) system (Cell Signaling, Danvers, MA, USA) and the film was developed for brand visualization. Densitometry analysis of expression bands of pCREB against the internal control of total CREB was performed and then the data were normalized with the data on day 1 in the Sham group. The final data were presented as the fold increase relative to the control.

Statistical analysis

Data were expressed as means ± SD. Statistical comparison was performed with analysis of variance (ANOVA) either by the Student–Newman–Keuls test or Dunn's comparison wherever appropriate. A p value of < 0.05 was considered as statistical significance.

Results

Effect of intrathecal administration of AIP on pain threshold

The MWT in the sham surgery animals did not change considerably throughout the experiment. However, 1 day after surgery, the MWT in the SNL group rats was significantly decreased by 60–70% up to post-operation day 14 ($p < 0.05$–0.01) when compared to the baseline (Fig. 1). Intrathecal administration of saline did not show any effect on the MWT changes. In contrast, API injection significantly delayed the onset of allodynia development for 3 days, which showed that the MWT was increased by 20% when compared to that in the SNL group ($p < 0.05$). Subsequently, the MWT returned to the same level as that in the SNL group (Fig. 1).

In the group of postoperative injection of AIP, the MWT was increased by 30% when compared to that in the saline injection group at half an hour after injection. The MWT gradually returned to the level comparable to that in the saline injection group 8 h after injection. This indicated that AIP administration after the establishment of mechanical allodynia was less effective than the preemptive treatment, in which its effect lasted for 3 days (Fig. 2).

pCaMKII expression

Compared with the sham operative rats, the expression of pCaMKII on the ipsilateral side of the dorsal horn in the SNL rats was considerably increased, while intrathecal AIP

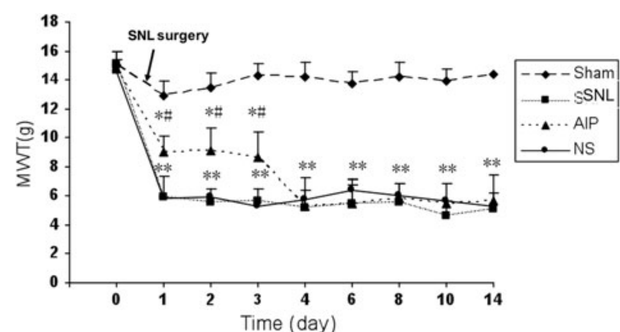

Fig. 1 Effects of the preemptive intrathecal injection of AIP on the change of the mechanical withdrawal threshold (*MWT*) in the rats with sham surgery (*Sham*) or sciatic nerve ligation (*SNL*) with or without AIP or normal saline (*NS*) injection. Results are means ± SD ($n = 8$). $^{*}p < 0.05$, $^{**}p < 0.05$ versus sham at the corresponding time point; $^{\#}p < 0.05$ versus NS group

but not saline injection led to a significant reduction of pCaMKII expression on post-experimental day 1 (Fig. 3a, b). These changes were also evident on post-experimental day 3. There was no statistically significant difference among these three treated groups (Fig. 3b).

pCREB expression

In line with the expression of pCaMKII, on western blot analysis similar patterns of changes in the expression of pCREB were found in the spinal cord, i.e., a decrease in expression on post-experimental day 1 but not on days 3 and 7 after AIP treatment. However, there was no change following saline injection (Fig. 4).

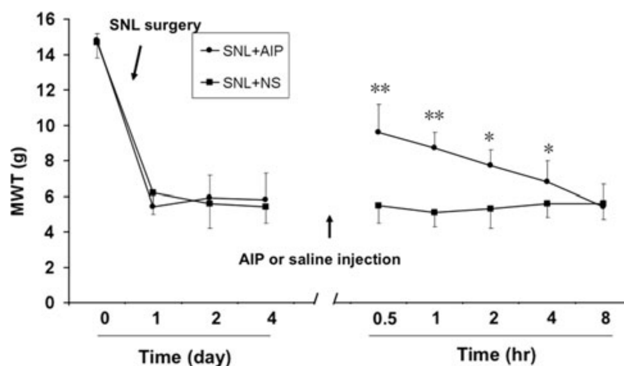

Fig. 2 Effect of intrathecal injection of AIP after surgery on the change of MWT in the rats with sciatic nerve ligation (*SNL*). Results are means ± SD ($n = 8$). *$p < 0.05$, **$p < 0.01$ versus normal saline (*NS*) injection at the corresponding time point

Discussion

This study demonstrated the involvement of CaMKII in the central sensitization of nociceptive dorsal horn neurons that follows partial sciatic nerve ligation. Administration of the CaMKII inhibitor, AIP, attenuated the pain threshold reduction induced by sciatic nerve injury, which provided evidence that CaMKII likely contributed to the behavioral changes associated with central sensitization. Our results also showed that pCREB was associated with CaMKII-modulated neuropathic pain development.

The multifunctionality of CaMKII, which is especially abundant in the nervous system, has been implicated in various neuronal functions, such as the modulation of neurotransmitter receptors and ion channels, gene expression, neurite outgrowth, and synaptic plasticity, including long-term potentiation and central sensitization [4, 13–16]. Under physiological conditions, CaMKII stays in an inactive form. However, once CaMKII is activated under pathological conditions, it mediates a variety of different cellular responses that convey signal transduction from the plasma membrane to the nucleus, thereby strengthening cellular responses to peripheral stimulation [17]. Recently, it has been shown that CaMKII is preferentially localized in pain-processing regions in the nervous system, such as the superficial laminae of the dorsal horn in the spinal cord and the dorsal root ganglia [18], which suggests that this enzyme may contribute to nociceptive processing. Indeed, it has been reported that CaMKIIa and pCaMKIIa were significantly increased in the spinal cord after the intradermal injection of capsaicin, and their role in central pain

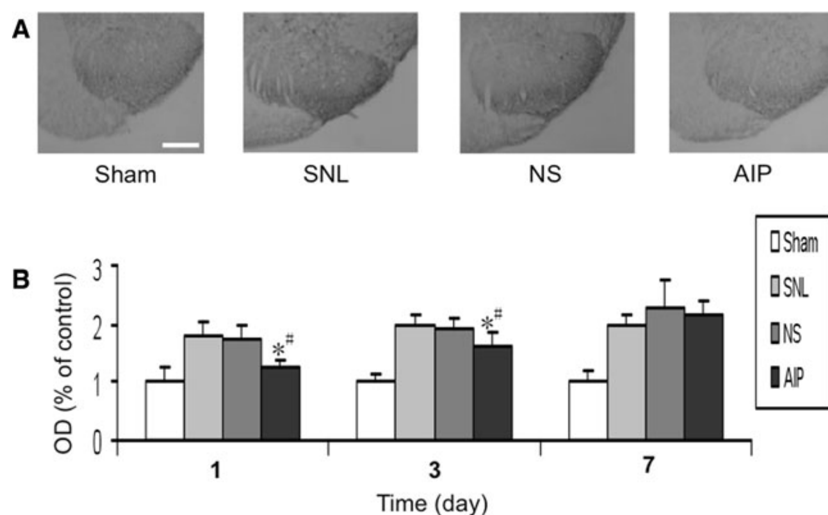

Fig. 3 Effects of the intrathecal injection of AIP on the expression of phosphorylated calcium/calmodulin-dependent protein kinase (pCaMK) II in the spinal dorsal horn in the rats with sham surgery (*Sham*) or sciatic nerve ligation (*SNL*) with or without AIP or normal saline (*NS*) injection on post-experimental days 1, 3, and 7. **a** Example microimages of spinal dorsal horn obtained 1 day after the experiment. *Bar* 100 μm. **b** Time course of the effects of the intrathecal injection of AIP on the expression of pCaMKII. Results are means ± SD ($n = 4$). *$p < 0.05$ versus SNL; $^{\#}p < 0.05$ versus SNL + NS

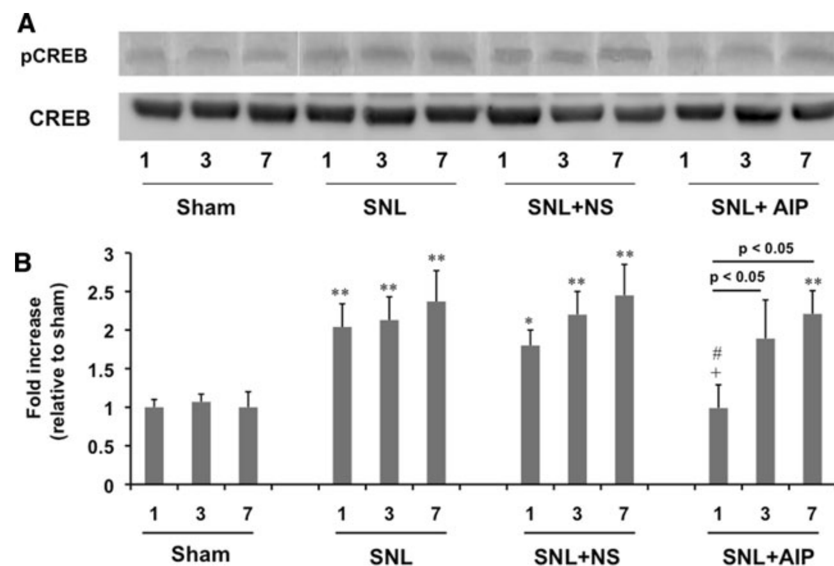

Fig. 4 Effects of the intrathecal injection of AIP on the expression of phosphorylated cyclic adenosine monophosphate (cAMP) responsive element binding protein (*pCREB*) against total CREB in the spinal dorsal horn in the rats with sham surgery (*Sham*) or sciatic nerve ligation (*SNL*) with or without AIP or normal saline (*NS*) injection on post-experimental days 1, 3, and 7. **a** The representative bands are from one of three independent experiments. **b** The fold increase of pCREB relative to total CREB. Results are means ± SD ($n = 4$). $^{*}p < 0.05$; $^{**}p < 0.01$ versus the corresponding time point in the Sham group; $^{\#}p < 0.01$ versus on day 1 of SNL; $^{+}p < 0.05$ versus on day 1 of SNL + NS

sensitization development has been well documented [4]. Our results showed that, compared with the normal controls, the pCaMKII-immunoreactive profile in the superficial dorsal horn on the ipsilateral side was largely increased after SNL. The upregulation was abolished by the pCaMKII inhibitor AIP but not by saline treatment.

The signaling pathways for nociceptive transduction are comprised of cascades of intracellular protein kinases and the phosphorylation of transcription factors including CREB; all of which have been reported previously to be involved in central sensitization processing [7, 8, 19]. Phosphorylated CREB may be involved in the downstream signal transduction cascade in several cellular pathways through the transcription regulation of nociception-related genes, such as the immediate early gene, *c-fos*, which reflects the activation of nociceptive neurons. A binding site for CREB was found in promoter regions including *c-fos*, c-jun, NK-1, Cox-2, and BDNF, which are involved in nociceptive modulation [7, 20–23]. In an in vitro preparation, the decreased phosphorylation of CREB was reported to be associated with reduced CaMK activity in the hippocampus [19, 24]. The results from our present study showed that significant upregulation of pCREB was found in the dorsal horn of the spinal cord after SNL; this result is in line with a previous study [25, 26], indicating that pCREB is an important modulator in the pathogenesis of neuropathic pain following nerve injury.

The change in pCREB regulation was in parallel with the upregulation of pCaMKII in the neuropathic pain state. These results may indicate that pCaMKII plays an important role in neuropathic pain development which is associated with the upregulation of its downstream effector, pCREB.

In the present study, we found that inhibiting pCaMKII significantly abolished CREB phosphorylation in the spinal cord. These results provide evidence that the activation of CaMKII participates in the phosphorylation of CREB during central sensitization development. However, the mechanism by which CaMKII regulates CREB phosphorylation, directly or indirectly, remains unclear. Further investigation of this aspect would expand our understanding of the role of CaMKII in triggering CREB phosphorylation leading to a neuropathic status. Nevertheless, the downregulation of pCREB expression directly via adenylate cyclase [25] or antisense oligonucleotide [26], or indirectly via a CaMKII inhibitor as demonstrated in the present study, attenuated neuropathic pain, which would suggest that the down-modulation of pCREB may have value for chronic neuropathic pain treatment.

In conclusion, our results indicate that CaMKII is important for the induction of central sensitization in spinal dorsal horn neurons in neuropathic pain. CaMKII plays a regulatory role in the maintenance of the phosphorylation of CREB protein in the spinal cord in response to peripheral noxious stimulation. These findings provide a likely explanation of how the activated intracellular kinase cascades convey signals into the nucleus during central sensitization in the spinal cord.

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